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Adherence of bile-isolated bacteria to the bile ducts mucosa as a pathogenic factor in the development of inflammatory lesions

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SUMMARY

Bacterial infection of the bile system appears to be an important factor in the formation of stones. In view of the hypothesis that strains of E. c. form an essential factor in infections of the bile ducts, an attempt has been made to determine the connection between infections of the bile ducts and the adherence of E. c. to the epithelium of the gallbladder. The research covered 148 patients operated electively for cholecystolithiasis (121), cholecystocholedocholithiasis (26) and recurrent lithiasis (1). In bile collected from the gallbladder in the course of the operation, E. coli strains were isolated. Cholangioscopy performed in 26 patients enabled the macroscopic evaluation and grading of inflammatory lesions of bile duct mucosa. The mucosa of the gallbladder was evaluated histologically. The adherence test was performed using homologous and heterologous strains of E. c. isolated from the bile of gallstone patients. The adherence occurred most frequently in the neck of the gallbladder (71–100%) in those patients in whom an infectious process of the bile ducts mucosa was endoscopically diagnosed. The adherence of bacteria to the epithelium of the gallbladder did not depend on the type of inflammation (acute, chronic).

INTRODUCTION

Gallstones, and particularly recurrent choledocholithiasis, continues to be a problem for clinicians, despite enormous progress in diagnostics and the application of new therapeutic methods. The pathogenesis of this disorder has not yet been determined.

Bacterial infection of the bile system appears to be an essential factor in the formation of stones. Such infections most often develop as a result of existing lesions, such as ischemia, compression, or cholestasis [1,2]. The infection may be ascending in character, from the duodenum through the common bile duct, by blood through arterial vessels, venous vessels and the portal system, or descending from the liver, through bacteria carried along with bile by lymphatic vessels, from inflammatory foci present in the adjacent organs, i.e. the stomach, duodenum or appendix [1]. It is assumed that one of the factors playing a role in the pathogenesis of gallstones is *Escherichia coli* [3,4], which belongs to the physiological flora of the large intestine [5]. *E. coli* can, however, be an etiological symptomatic factor in infections with very diverse localization of the disease process (infection of wounds, septicemia, abscess of the liver, urinary tract infections). In the pathogenesis of bacterial infections, great significance is assigned to the adherence properties of bacteria [6,7], i.e. the permanent attachment of the microorganism to the cells of the host. One of the characteristic features of the majority of *E. coli* strains is adherence to the

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cells of the intestinal epithelium, which causes lasting damage [8,9]. Integrity is lost due to their adherence, and with it the barrier function of the epithelial cells. Changes are brought about in the structure of the skeleton of the eucaryot cell. The pathogenicity of some strains of *E. coli* is associated with the fact that it has eaeA, eaeB, and sep (cfm) genes, located on the bacteria chromosome inside the 35kb region, known as the LEE (locus of effacing *E. coli*) [10]. This area does not occur in normal physiological flora.

The basic topic of the present study is the relationship between the adherence of *E. coli* to the epithelium of the gallbladder and infectious lesions of the mucosa of the gallbladder and the bile ducts in gallstone patients.

MATERIAL AND METHODS

The research covered 148 patients with gallstones (116 women and 32 men, see Figure 1), undergoing elective surgery at the First Clinic of General Surgery Jagiellonian University in Cracow. In this group:

- 121 patients had cholecystolithiasis;
- 26 patients had cholecystolithiasis and choledocholithiasis;
- 1 patient had recurring lithiasis.

Bile for bacteriological testing was collected by puncture of the gallbladder prior to cholangiofiberoscopy. Cholangioscopy, performed with a flexible instrument manufactured by Olympus, enabled the macroscopic evaluation of the state of advancement of infectious lesions of the mucosa of the bile ducts.

The nature of the infectious lesions of the mucosa of the gallbladder was evaluated histologically.

1. Methodology of bacteriological determination

The bile collected in the course of the operation was inoculated onto a basic medium for aerobic bacteria culture and onto a MacConkey medium, selectively differentiating for bacteria belonging to the family *Enterobacteriaceae*.

The Gram-negative bacteria were assigned to a species using an API 20E enzymatic test manufactured by API (11). The *E. coli* strains isolated from the bile were preserved on the medium for aerobic bacteria culture by using Tryptic Soy Broth with the addition of 2% agar, at a temperature of 4°C.

2. Origin and selection of strains for adherence test

The adherence test according to Wäisänen et al. [12] was performed using the strains of *Escherichia coli* isolated from the bile of gallstone patients.

In the group of patients in whose bile the presence of *E. coli* was confirmed, the test of adherence to the epithelium of the gallbladder was performed with homologous strains.

In the other groups of patients, in whose bile the presence of *E. coli* was not confirmed, the test of adherence to the epithelium of the gallbladder was performed with a heterologous strain of *E. coli*, which was chosen from among 15 previously selected strains from the pilot research project.

In order to designate the heterologous strains, a preliminary test of the adherence of these strains to the epithelium of the gallbladder was performed, and then two strains displaying significant (+++) adherence were selected.

The adherence test was performed first with one, and then the other selected test strain, in order to avoid erroneous results caused by the possibility of the loss of certain biological properties of the strains.

10 strains of *E. coli* isolated from urine and 10 strains of *E. coli* isolated from diarrheic stool were used as antagonistic groups for the adherence test.

3. Adherence of strains of Escherichia coli to the epithelium of the gallbladder

The epithelium used for the test of *E. coli* adherence came from the neck, corpus, and fundus of the gallbladder excised during surgery.

5 μm sections of the epithelium, cut in a frozen state using a microtome, were placed on a basic slide, dried, and kept in a freezer at a temperature of –18°C.

In a certain group of patients (10 patients selected at random) three slides were prepared, of which one served for the test of adherence of *E. coli* isolated from bile, and the other two for the adherence test with strains coming from diarrheic stool and urine.

The adherence test was performed with a suspension of the corresponding strain of *E. coli*.

Table 1. Dependence of Escherichia coli adherence to the epithelium of the gallbladder on the degree of progression of infectious lesions of the mucosa of the bile ducts, evaluated macroscopically (cholangiofiberoscopy)

Condition of the mucous membrane		Epithelium of the gallbladder									Total
		Neck			Corpus			Fundus			
		–	+	++	–	+	++	–	+	++	
no lesions n=4	n	4	0	0	2	2	0	3	1	0	4
	%	100	0	0	50.0	50.0	0	75.0	25.0	0	16.0
infectious lesions of the	n	6	12	3	13	8	0	14	7	0	21
mucosan=21	%	28.6	57.1	14.3	61.9	38.1	0	66.7	33.3	0	84.0
Statistical significance		Fisher exact test p⩽0.05			Fisher exact test NS			Fisher exact test NS			

Legend: no adherence (–); degree of adherence in limits from (+) to (++)

Table 2. Adherence of Escherichia coli strains to the epithelium of the gallbladder in dependence on the types of infectious lesions confirmed in patients by histological evaluation.

Condition of the mucous membrane		Epithelium of the gallbladder												Total
		Neck				Corpus				Fundus				
		–	+	++	+++	–	+	++	+++	–	+	++	+++	
chronic	n	36	72	24	3	44	81	10	0	79	51	5	0	135
cholecystitis n=135	%	26.7	53.3	17.8	2.2	36.2	60.0	7.4	0	58.5	37.8	3.7	0	91.2
acute infectious	n	3	5	5	0	8	5	0	0	9	4	0	0	13
lesions n=13	%	23.0	38.5	38.5	0	61.5	38.5	0	0	69.2	30.8	0	0	8.8
Statistical significance		Fisher exact test NS				Fisher exact test nearly sig.				Fisher exact test NS				

The density of the suspension was evaluated spectrophotometrically, on the basis of the extinction value, the value of which, E = 0.75, corresponded to a density of 10^9 bacterial cells in 1 ml of phosphate buffer (PBS) with a pH of 7.1.

A buffered fluorescein solution was added to 3 ml of the bacterial suspension thus obtained. Then, after centrifugation of sediments, the suspension was irrigated three times with a Tween 20 buffer (30 ml PBS, pH = 7.1, containing 0.05% Tween 20), suspended in a buffered solution of Tween 10 and 1% bovine serum, and placed on the previously prepared gallbladder epithelium. After 40 minutes of incubation, the solutions were rinsed three times and inspected under a fluorescent microscope (magnification 2500 x).

The degree of adherence was specified semi-quantitatively, on the basis of the number of fluorescent bacteria visible in the microscopic field of view, as follows:

+++ uncountable number of bacteria
++ more than 10 bacteria in the field of view
+ up to 10 bacteria in the field of view
– no bacteria in the field of view

4. Statistical processing

In order to test the differences that occurred among the analyzed groups, the non-parametrical chi-squared test was used (3).

RESULTS

1. Adherence of *E. coli* strains isolated from bile to gallbladder epithelium

Cholangioscopy is a modern endoscopic technique, introduced in the late 1960s and early 70s, used to examine the bile ducts with a flexible instrument during a surgical procedure. Its application increases the possibility of discovering stones in the bile ducts. Using this technique, it is also possible to evaluate the status of the mucosa of the bile ducts, previously specified only on the basis of clinical symptoms and the results of laboratory tests.

Intraoperative cholangiofiberoscopy was performed on 25 of the tested patients. Table 1 shows a comparison of the adherence of *E. coli* in the groups of patients without infectious lesions (4 patients) and with infectious lesions of the mucosa of the bile ducts (21 patients).

Table 3. Comparative analysis of the adherence of homologous and heterologous strains of E. coli to the epithelium of the gallbladder.

Adherence	Homologous strain				Heterologous strain				Statistical significance
	+		–		+		–		
	n	%	n	%	n	%	n	%	
neck	37/37	100.0	0/37	0	72/111	64.9	39/111	35.1	Fisher exact test $p \leqslant 0.001$
corpus	34/37	91.9	3/37	8.1	62/111	55.8	49/111	44.2	Fisher exact test $p \leqslant 0.001$
fundus	12/37	32.4	25/37	67.6	48/111	43.2	63/111	56.8	Fisher exact test NS

Table 4. Comparison of the adherence of E. coli strains isolated from stool and urine to the epithelium of the gallbladder.

Adherence	Strains derived from stool				Strains derived from urine				Statistical significance
	+		–		+		–		
	n	%	n	%	n	%	n	%	
neck	2/10	20.0	8/10	80.0	3/10	30.0	7/10	70.0	Fisher exact test NS
corpus	1/10	10.0	9/10	90.0	0/10	0	10/10	0	Fisher exact test NS
fundus	0/10	0	10/10	100.0	0/10	0	10/10	0	NS

Legend: no adherence (-); adherence (+)

Table 5. Comparison of the adherence of E. coli strains derived from stool and urine to the epithelium of the gallbladder.

Adherence	Bile				Diarrhea		–Urine	
	Own strain		Test strain					
	n	%	n	%	n	%	n	%
neck	37/37	100.0	72/111	64.9	2/10	20.0	3/10	30.0
corpus	34/37	91.9	62/111	55.8	1/10	10.0	0/10	0
fundus	12/37	32.4	48/111	43.2	0/10	0	0/10	0

It was discovered that the phenomenon of adherence occurred most frequently in the neck of the gallbladder (71.4%) in those patients in whom an infectious process of the bile ducts was simultaneously diagnosed.

Statistically significant differences in the frequency of adherence of *E. coli* to the epithelium of the neck of the gallbladder were observed between the group of patients without infectious lesions and those with infectious lesions of the bile duct mucosa. No statistically significant differences (NS) were discovered, however, in the corpus and fundus of the gallbladder. It can be inferred from a quantitative analysis that intensified adherence (++) occurred in 3 subjects (14.3%) in the neck of the gallbladder.

Table 1 (along with Figs. 1–3, which provide a graphic illustration of the table) present the results pertaining to *E. coli* adherence to the epithelium of the neck, corpus, and fundus of the gallbladder.

Table 2 presents *E. coli* adherence to the epithelium of the gallbladder in relation to the type of infectious lesions confirmed histologically.

In the tested group of 148 patients, 135 had a diagnosis of chronic cholecystitis, and 13 were diagnosed with acute infectious lesions of the gallbladder. From Table 2 it follows that the adherence of bacteria to the epithelium of the gallbladder did not depend on histologically evaluated infectious lesions. The frequency was approximately the same in the case of acute and chronic lesions.

The differences in the frequency of adherence to the epithelium of the neck and fundus of the gallbladder in the case of cholecystitis chronica and acute infectious lesions were not statistically significant (NS).

The data from Table 2 have been presented graphically in Figs. 4, 5, and 6.

As it can be seen in Table 2, the presence of *E. coli* was confirmed in the bile of 37 from among the 148 tested patients. The adherence test was performed in these patients with a homologous strain. In the remaining 111 cases, appropriately selected strains of *E. coli* were used for adherence.

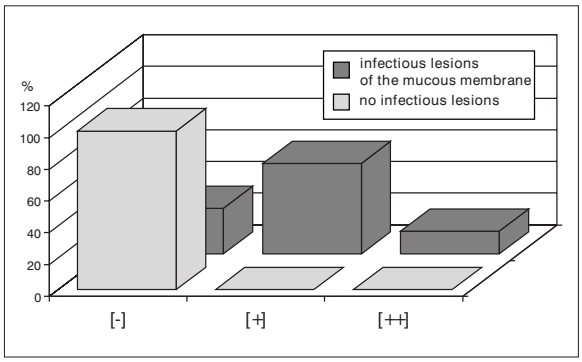

Figure 1. Adherence of *E. coli* to the epithelium of the neck of the gallbladder and infectious lesions of the mucosa of the bile duct.

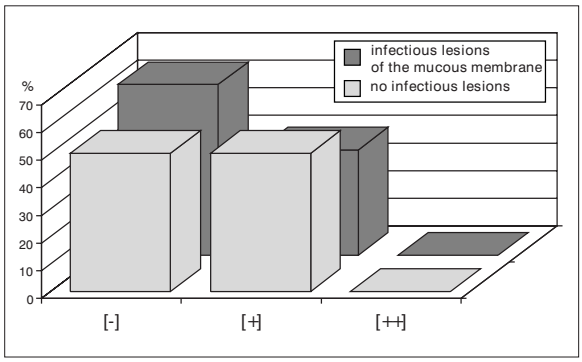

Figure 2. Adherence of *E. coli* to the epithelium of the corpus of the gallbladder and infectious lesions of the mucosa of the bile ducts.

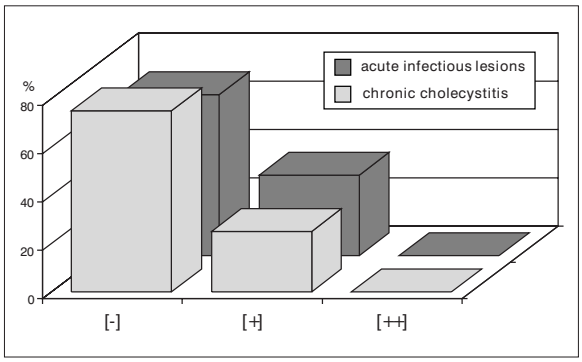

Figure 3. Adherence of *E. coli* to the epithelium of the fundus of the gallbladder and infectious lesions of the mucosa of the bile ducts.

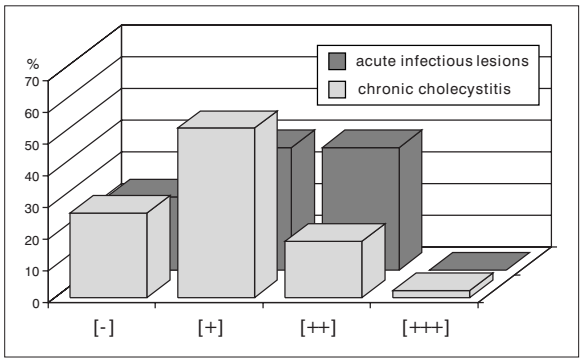

Figure 4. Adherence of *E. coli* to the epithelium of the neck of the gallbladder and infectious lesions of the gallbladder.

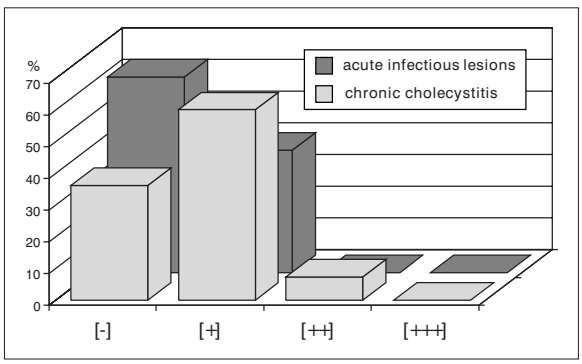

Figure 5. Adherence of *E. coli* to the epithelium of the corpus of the gallbladder and infectious lesions of the gallbladder.

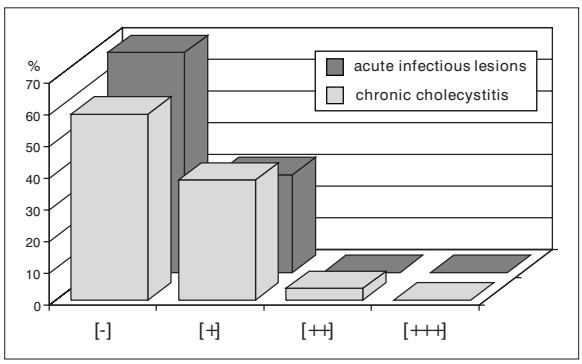

Figure 6. Adherence of *E. coli* to the epithelium of the fundus of the gallbladder and infectious lesions of the gallbladder.

In Table 3, the frequency of adherence to the epithelium of the neck, corpus, and fundus of the gallbladder is compared in the group of 37 patients with homologous strains and in the group of 111 patients with a heterologous strain.

In both tested groups, the highest frequency of adherence was found to the epithelium of the neck of the gallbladder (100%, 64.9%), and the lowest to the epithelium of the fundus (32.4%, 43.2%). In the case of adherence of homologous strains of *E. coli* to the epithelium of the neck, adherence was 100%.

Statistically significant differences were indicated between the adherence of homologous and heterologous strains of *E. coli* to the neck and corpus of the gallbladder.

2. Adherence to the epithelium of the gallbladder of *E. coli* strains isolated from diarrheic stool and urine

Table 4 presents a comparison of adherence of strains of *E. coli* isolated from diarrheic stool and urine to the epithelium of the gallbladder.

No statistically significant differences were discovered between the adherence of these strains to the epithelium of the neck, corpus, and fundus of the gallbladder. Most often, both in the case of *E. coli* strains isolated from diarrheic stool and those isolated from urine, adherence was discovered in the neck of the gallbladder (20%, 30%). No adherence of these strains to the fundus of the gallbladder was detected.

As it can be seen in Table 5, where a comparison is made between the adherence of strains of *E. coli* derived from bile, diarrheic stool, and urine to the epithelium of the gallbladder, adherence took place most frequently (100%) to the epithelium of the neck of the gallbladder, in the case of homologous strains of *E. coli* originating from the patient's bile.

DISCUSSION

The presence of bacteria in bile is a very serious clinical problem. Infections of post-surgical wounds, septic complications, and other complications are more frequently discovered in patients with a positive bile culture. According to Mansiion [13], abscesses of post-surgical wounds was discovered in 70% of those patients whose bile cultures were positive. The same strains were cultivated from bile collected post-operatively and from wound secretions.

In 1969, Liedberg [14] observed that the motor activity of the gallbladder changes as a result of bacterial infection. This leads to bile stasis, and later to the development of gallstones [15].

Advanced infectious lesions of the bile ducts are accompanied by a widening of the common hepatic and bile duct. Such widening is visible in radiological examination, and points to impairment of the motor activity of the extrahepatic bile system. This would seem to justify the reports of the frequent occurrence of primary choledocholithiasis in patients with infected bile. As it can be inferred from the research conducted by various authors, the incidence of infected gallbladder bile is about 25%, while in patients with choledocholithiasis the figure is 75%. In the cases where examination in the course of surgery confirms infectious lesions of the mucosa of the bile ducts, this percentage may reach as high as 90%. In patients with recurring gallstones, bacterial infection is confirmed in 84–90% of cases [16,17,18].

Constrictions of the bile ducts caused by infectious lesions and infections, and by stasis of bile, have a decisive impact on the formation of stones [19]. The mechanisms leading to the formation of gallstones are important in view of the fact that in virtually every case pigmentary substances constitute the kernel of crystallization for cholesterol [20].

The bacteria present in bile can modify its composition in two ways: directly, through their effect on the lipid composition, or indirectly, by changing the motor activity of the extrahepatic bile ducts. Both mechanisms lead to changes in the composition of the bile, and as a result to the appearance of lithogenic bile.

The most essential complication of bile infection and stasis, however, is the recurrence of lithiasis. The essence of this recurrence involves the persistence of infectious lesions, infections, and inhibited passage of bile, symptoms which do not recede after the stone is removed. Patients with choledocholithiasis, and especially in its recurrent manifestation, more often have lithogenic bile, and the excision of the gallbladder does not lead to the improvement of its lipid composition. These patients continued to be threatened with the development of lithiasis.

The research done to date on infections of the bile ducts has shown that *E. coli* most often occurs in the bile of patients with gallstones [21,22].

In view of the confirmation that strains of *E. coli* form an essential factor in infections of the bile ducts, it has been attempted to indicate the connection between infections of the bile ducts and the adherence of *E. coli* to the epithelium of the gallbladder.

In the set of factors that influence the human organism, the structures associated with the bacterial cell should be taken into consideration: somatic antigens, capillaries, cilia, and fimbriae, as well as the substances excreted from the cell, including toxins, hemolizines, enzymes, and other metabolites.

Recently a considerable amount of attention has been aroused by the adherence properties of bacteria, to which an essential role has been assigned in the process of colonization and development of infection [23,24]. To date, the structure and role of the adherence fimbriae of K88 and K99, CFA I and CFA II, as well as CFA III and IV, have been described [25–27]. The enterotoxic strains of *E. coli*, which are etiological factors in human and animal enteritis, have such fimbriae. The fimbriae P of Escherichia coli are also known to have a role in urinary tract infections.

To date, however, there has been no description or characterization of the pathogenic factors of *E. coli* that may contribute to the pathomechanism of bile duct infections. The fact that these bacteria are held in the bile, that they can colonize the bile ducts, and that in this way they bring about the spread of infection, leads to the supposition that they possess hitherto unknown adherence factors making it possible for them to adhere to mucosa.

The present research has confirmed the existence of a certain mechanism of *E. coli* adherence to the epithelium of the gallbladder. It remains unclear why the largest percentage of bacteria adhere to the epithelium of the gallbladder neck (100% adherence with a homologous strain, 64.9% with the heterologous), and the smallest percentage adhere to the epithelium of the fundus (32.4% with a homologous strain, 43.2% with a heterologous strain). From the comparison of *E. coli* adherence in the groups of patients without infectious lesions and those with infectious lesions of the mucosa of the bile ducts, confirmed post-operatively by cholangioscopy, it can be inferred that this phenomenon occurs most often in the neck of the gallbladder in the patients simultaneously diagnosed with an infectious process in the bile ducts. The fact that no adherence at all was discovered in the group of patients without infectious lesions in the bile ducts indicates that a role may be played in this process by adherence factors other than fimbriae. These may be host-related factors.

Structural proteins, such as fibronectine or laminine, take part in the adherence of bacteria to eucaryot cells. Fibronectine, a glycoprotein, serves as a so-called 'acquired receptor', formed from the substances produced by the organism of the host, but not directly by the cells on which these receptors occur. It forms a bridge connecting both cells. This may be one of the mechanisms (methods) of bacteria adhesion to specified tissues. The role of fibronectine has been described as a receptor on the surface of mucosa cells for many Gram-positive bacteria, such as Staphylococcus aureus or Streptococcus pyogenes. For example, fibronectine-specific receptor spots have been identified on the surface of Staphylococcus aureus [28]. Fibronectine combines with the cells of Gram-positive bacteria in a domain localized close to the N-terminal amino acid. On eucaryot cells, the place of fibronectine binding is localized in the middle of the polypeptide chain [23]. The fibronectine may also combine with Gram-negative bacteria, such as *E. coli*. This glycoprotein may be of significance in the mechanism of *E. coli* adherence to the mucosa of the gallbladder. It has been discovered to occur in the place where the mucosal cells of the intestine are joined. Its presence has also been discovered in the mucosa of the rectum [29].

In the case of the secretion by bacteria of cytolytic toxins, the cells of the mucosa undergo lysis, and the basal mucosa is exposed in which laminine is found (M-900 000 glycoprotein). Laminine mediates the process of adherence of such bacteria as *E. coli*, Streptococcus pyogenes, or Staphylococcus aureus to eucaryot cells [30,31].

At the present stage of research, there can only be debate on the mechanism of *E. coli* adherence to the mucosa of the bile ducts. The adherence process in which laminine participates should be taken into consideration, since specific adherence is in fact rendered impossible by infectious lesions of the mucosa in patients with gallstones, confirmed macroscopically and histologically, and leading to desquamation of the mucosa, infectious infiltrations, and even degenerative lesions and gangrene [32]. The mucous substances that play a role in the crystallization and growth process of cholesterol stones may also take part in the adherence process [33]. The role of mucus in the adherence of Pseudomonas aeruginosa in mucoviscidosis is known and has been described [34]. The quantity of mucous substances increases during bacterial infections, which, as confirmed in animal experiments, precedes the development of lithiasis [35].

In the present paper, adherence has also been analyzed in terms of the types of infectious lesions confirmed histologically. It was discovered that bacteria adherence to the gallbladder mucosa does not depend on the type of histologically evaluated infectious lesions. The frequency of adherence is similar in the case of acute and chronic lesions.

The difference between the frequency of adherence of the tested strains of bile-derived *E. coli* to the mucosa of the gallbladder, and the frequency of adherence of *E. coli* isolated from diarrheic stool and urine, supports a distinction between these strains (Table 5).

CONCLUSIONS

1. The high frequency of *E. coli* adherence in the group of patients with infectious lesions in the mucosa of the bile ducts suggests non-specific adherence, in which acquired receptors take part, serving as a bridge between the bacterial adhesion and the receptor located in the deeper layers of the mucosa. The ongoing infectious lesions lead to desquamation of the mucosa, infiltrations, and even degenerative lesions.

2. The discovery of adherence confirms the hypothesis that infectious lesions of the bile ducts may be the result of the presence of bacteria.

3. The exact recognition of the receptor participating in adherence of *E. coli* to the mucosa of the bile ducts, and its blockade, may contribute in the future to the prevention of the formation of recurrent stones.

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